Detecting Three Common Drugs in Horses:

*Frusemide, betamethasone and triamcinolone acetonide*

by


N.J. Basgallop, G. Noble, B. Turco, M.N. Sillence, A.R. McKinney and A.M. Stenhouse

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Researcher Contact Details
Dr John H. Vine
Racing Analytical Services Ltd
400 Epsom Road
Flemington  Vic 3031
AUSTRALIA

03 9376 6760
03 9376 6875
jvine@rasl.com.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON  ACT  2600
PO Box 4776
KINGSTON  ACT  2604

Phone:  02 6271 4100
Fax:  02 6271 4199
Email:  rirdc@rirdc.gov.au.
Web:  http://www.rirdc.gov.au

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Foreword

The purpose of this research was to study the absorption, distribution, metabolism and excretion of three therapeutic substances – frusemide, betamethasone and triamcinolone acetonide, commonly used by veterinarians during the preparation of horses for competition in the Thoroughbred and Standardbred industries as well as in high level equestrian events.

Frusemide is a ‘high ceiling’ diuretic commonly used in the management of exercise induced pulmonary haemorrhage in racehorses. Betamethasone and triamcinolone acetetonide are corticosteroid drugs used for intra-articular treatment and/or management of horses with degenerative joint disease.

Several of the analytical procedures used to detect these drugs in urine samples from horses in competition horses are about to change. These changes may alter the detection periods of these commonly-used drugs. The proposed project provides reliable and up-to-date drug excretion data for frusemide to practising veterinarians in a biologically meaningful number of horses. Pilot studies involving corticosteroid drugs have provided preliminary data on likely detection periods. This constitutes a first step in providing information on the responsible use of intra-articular corticosteroids which is consistent with animal welfare considerations and with the racing industry’s rules on the use of medications in racehorses.

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This report, an addition to RIRDC’s diverse range of over 1600 research publications, forms part of our Horse R&D Program, which aims to assist in developing the horse industry and enhancing its export potential.

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Abbreviations

APCI  Atmospheric pressure chemical ionisation
ARFL  Australian Racing Forensic Laboratory
AUC  Area under the concentration curve
Cmax  Maximum concentration
CI  Clearance
CSU  Charles Sturt University
Da  Daltons
ESI  Electrospray ionisation
HC  Hydrocortisone
HESI  Heated electrospray ionisation
HPLC  High performance liquid chromatography
IA  Intra-articular
IV  Intravenous
Kel  Terminal phase constant
LC  Liquid chromatography
LCMS  Liquid chromatography mass spectrometry
LC/MS/MS  Liquid chromatography/mass spectrometry/mass spectrometry
LOD  Limit of detection
LOQ  Limit of quantitation
MDL  Method detection limit
MRM  Multiple reaction monitoring
MS/MS  Mass spectrometry/mass spectrometry
NATA  National Association of Testing Authorities, Australia
OHTA  Hydroxytriamcinolone acetonide
PV  Peri-vascular
ERO  Reverse osmosis
SG  Specific gravity
SRM  Selective reaction monitoring
TA  Triamcinolone acetonide
T1/2  Terminal half-life
Tmax  Time of maximum concentration
TSQ  Triple stage quadrupole
V  Volume of distribution
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Executive Summary

What is the report about?
The purpose of this research was to study the absorption, distribution, metabolism and excretion of three common veterinary pharmaceuticals, (1) frusemide, which is a diuretic and the corticosteroids, (2) betamethasone and (3) triamcinolone acetonide. These substances are commonly used by veterinarians during the preparation of horses for competition in the Thoroughbred and Standardbred industries, as well as in high level equestrian events. The data obtained will be used to provide reliable detection times which meet the needs of racing industry regulators, practising veterinarians and the owners and trainers they service.

Background
Frusemide is a high ceiling diuretic commonly used in the management of exercise induced pulmonary haemorrhage in racehorses. Despite its long-standing use, there is little reliable data on its urinary excretion which is directly applicable to drug testing in Australia. Betamethasone and triamcinolone acetonide are corticosteroid drugs with profound anti-inflammatory properties and are widely used in the treatment of racing injuries. In the last few years, they have been increasingly administered intra-articularly (IA) rather than by intravenous (IV) or intramuscular (IM) routes. There is little or no information on the excretion and detection of these drugs after IA administration and hence there is no data upon which veterinarians can base informed decisions on the use of these drugs.

Methods
Robust and properly validated detection methodology has been applied to the measurement of these three drugs in blood and urine. Methods based on gas chromatography mass spectrometry and liquid chromatography mass spectrometry have been developed to ensure that the data generated are as definitive and reliable as possible and that experimental protocols are aligned with those being used to carry out related studies in other countries. This ensures that data will be transferable and enables the range of drugs for which reliable excretion data are available to be greatly extended.

Results
It has been shown that after IA administration, betamethasone may be detected for up to three days in urine, albeit at very low concentrations. There is no evidence of any significant retention of the drug in synovial fluid and hence it is unlikely that its systemic persistence and excretion in urine is prolonged by this route of administration. This is also supported by the noted suppression of endogenous hydrocortisone production which is of only two-three days duration.

The study on frusemide has shown that this drug may be reliably detected up to 48 hours in blood and urine. Data collected on the volume of urine voided and urine specific gravity may be interpreted to suggest that the diuretic effect would not extend beyond that period. Therefore, a 48 hour post administration detection time can be recommended with a high level of confidence, given that inter-subject variability was relatively small.

The study of triamcinolone acetonide administered by IA injection has demonstrated that this drug and its metabolite can be detected for up to 130 (5.5 days) and 170 (7 days) hours respectively, provided that methods of appropriate sensitivity are applied. The detection times are roughly equivalent to the time for which endogenous hydrocortisone is suppressed suggesting that they may also correlate with the duration of action of the drug. Inter-subject variability was relatively small with respect to the excretion of the drug and its metabolite after 60 hours. However, there was considerable variation in the rate at which endogenous hydrocortisone levels came back to pre-administration values.
**Implications**
This data can now be used by veterinarians to make more informed decisions about the treatment of horses, particularly in the weeks or days leading up to a race. The data will also assist the racing authorities to draw conclusion about purported treatment regimens in the event that one of these drugs is detected in a pre- or post-race sample.

**Recommendations**
We suggest that studies of this type continue so that similar relevant data can be generated for a realistically wide range of drugs commonly used for therapeutic purposes in race and competitive horses.
Introduction

In recent years the aim of the racing industry regulators to ensure “drug-free” competition and the need for veterinarians to properly treat racing injuries have increasingly come into conflict. The development of more sensitive analytical methods, necessary to detect today’s sophisticated “go-fast” drugs, may result in the detection of miniscule and pharmacologically irrelevant quantities of genuine therapeutic agents. The detection of such materials inevitably and in some cases unjustifiably reflects poorly on the racing industry and its participants. Reputations can be ruined and severe financial consequences may result from a trace presence of a relatively innocuous veterinary medication.

The purpose of this research is to study the absorption, distribution, metabolism and excretion of three common veterinary pharmaceuticals. The data obtained will make possible the determination of reliable detection times which meet the needs of racing industry regulators and practising veterinarians.

Three drugs were studied in this research; frusemide, which is a diuretic and the corticosteroids, betamethasone and triamcinolone acetonide. These substances are commonly used by veterinarians during the preparation of horses for competition in the Thoroughbred and Standardbred industries, as well as in high level equestrian events.

Frusemide is a ‘high ceiling’ diuretic commonly used in the management of exercise induced pulmonary haemorrhage in racehorses. Despite its long-standing use, there is little reliable data on its urinary excretion which is directly applicable to drug testing in Australia. The research we undertook examined the excretion and detection of frusemide in 10 horses using methodology which will provide an absolute measurement of drug concentration. The analytical approach (vide infra) will be consistent with that used in similar studies of other drugs currently underway in Europe. This was done to ensure that data is reliable and transferable.

Betamethasone and triamcinolone acetonide are corticosteroid drugs with profound anti-inflammatory properties and are widely used in the treatment of racing/athletic injuries. In the last few years they have been increasingly administered intra-articularly rather than by intravenous or intramuscular routes. There is little or no information on the excretion and detection of these drugs after IA administration and hence there is no data upon which veterinarians can base informed decisions on their use in this manner. The betamethasone research was intended to be a pilot study to provide some initial guidance on likely detection times. It was a limited study owing to the numbers of horses available, the sample types and Animal Ethics constraints. Its results, however, will assist in determining whether further, more detailed studies are warranted.
Methodology

The metabolism and excretion of betamethasone sodium phosphate and betamethasone acetate in the horse after intra-articular injection

Introduction
The use of corticosteroids administered by intra-articular injection has increased in popularity in recent years. This route of administration, directly into the site of action, allows the use of low doses of corticosteroids which results in relatively low urine concentrations compared with systemic administrations.

Recent studies have shown that IA injection of corticosteroids produces profound and long lasting suppression of endogenous hydrocortisone (Popot et al. 2003; Teale et al. 2004). This may be interpreted to suggest that these drugs either persist at the site of injection for long periods or that the effect of the drug administered in this way persists long after the drug has been excreted.

Celestone Chronodose® is a preparation containing betamethasone partially as the water soluble sodium phosphate ester and partially as the almost insoluble acetate ester. It is claimed by the manufacturer that sustained activity is provided by the slightly soluble acetate ester which acts as a repository and slows the rate of absorption (MIMS Australia 2006). Celestone Chronodose® is thought to be widely used, most commonly by IA injection, up to three days prior to racing or competition. Betamethasone is rarely detected in post-race urine samples.

This study was designed to examine the metabolism, distribution and excretion of the betamethasone esters in Celestone Chronodose® following IA injection into the carpi of two horses. Samples of blood, urine and synovial fluid were collected and analysed to determine the relative concentrations of betamethasone, betamethasone acetate and betamethasone sodium phosphate. Hydrocortisone concentrations were also measured to determine the degree of correlation between its concentration and those of the three forms of betamethasone.

Materials and methods
Celestone Chronodose® was obtained from Schering-Plough (Sydney, Australia). Betamethasone, betamethasone acetate, betamethasone sodium phosphate, hydrocortisone and triamcinolone acetonide were from Sigma Aldrich (Sydney, Australia) and d4-hydrocortisone was from Cambridge Isotopes Ltd (Andover, USA). Hyonate (10mg/mL sodium hyaluronate) was obtained from Bayer Australia Ltd (Sydney, Australia). 20% w/w bovine serum albumin was from ICP Bio Ltd (Auckland, New Zealand) and the Beckman High Calibrator was from Beckman Coulter (Sydney, Australia).

Administration and Sample Collection
Pre-administration, urine, blood and synovial fluid were collected, then Celestone Chronodose® (betamethasone sodium phosphate 3.9mg/mL and betamethasone acetate 3mg/mL; equivalent to 5.7mg/mL betamethasone) was administered as 1mL doses by IA injection into each of the radial carpal joints of two horses. Blood and urine samples were collected at 1, 3, 5, 8, 11 hours after administration and then at 1, 2, 3, 4, 6, 8, 10 and 13 days. Synovial fluid samples (approximately 100µL) were collected daily from one carpus of each horse, alternating between sides on successive days at 1, 2, 3, 4, 6, 8, 10 and 13 days. Immediately after collection blood samples were centrifuged (3000rpm, 20 min) and the plasma divided up into 4 x 1mL portions stored in plastic vials at –20°C. Urine and synovial fluid samples were stored untreated at –20°C.
**Preparation of “Synthetic” Plasma and Synovial Fluid**

A synthetic plasma surrogate which did not contain any hydrocortisone was made by diluting 1 part of a 20% solution of bovine serum albumin (w/v) with 4 parts of the Beckman High Calibrator. A synthetic synovial fluid surrogate which did not contain any hydrocortisone was made by diluting the plasma surrogate above 1:1 with water and then mixing 2 parts of this diluted plasma with 1 part of hyaluronic acid solution (10mg/mL).

**Extraction of Urine and Plasma**

Urine and plasma samples were extracted using Bond-Elut C18 columns (3cc/200mg; Varian, Australia). Columns were conditioned with methanol (2mL) and water (2mL) then loaded with urine (3mL) or plasma (1mL diluted to 2mL with water). The column was washed with 1% acetic acid (2mL) and eluted with dichloromethane : ethyl acetate : methanol (4:1; 2mL) and methanol (2mL). The organic eluates were combined, evaporated to dryness and reconstituted in 1% acetic acid : methanol (40:60 ; 200µL) for analysis by LCMS.

Calibration graphs covering the range 2.5 – 40 ng/mL for betamethasone and 2.5 – 40ng/mL for hydrocortisone in urine were prepared in steroid-stripped urine (Teale et al. 2004). Quality control spiked urines were prepared at 10ng/mL for betamethasone and at 10ng/mL for hydrocortisone. Calibration graphs covering the range 0.1 – 10ng/mL for betamethasone and 12.5 – 100ng/mL for hydrocortisone in plasma were prepared in the ‘in-house’ artificial plasma. Quality control spiked plasmas were prepared at 1ng/mL for betamethasone and at 25ng/mL for hydrocortisone. The internal standards were d4-hydrocortisone (at 200ng/mL in urine and 50ng/mL in plasma for hydrocortisone) and triamcinolone acetonide (at 20ng/mL in urine and 7.5 ng/mL in plasma, for betamethasone).

**Extraction of Synovial Fluid**

Synovial fluid samples were extracted using C18 Omix pipette tips (Varian, Australia). Omix tips were conditioned with methanol (2 x 100µL) and water (2 x 100µL) then loaded with synovial fluid (100µL). The tips were washed with water (100µL) and eluted with dichloromethane : ethyl acetate (4:1; 2 x 100µL) and methanol (2 x 100µL). The combined organic eluates were evaporated to dryness and reconstituted in 1% acetic acid : methanol (40:60; 200µL) for analysis by LCMS.

Calibration graphs covering the ranges 12.5 – 100ng/mL for hydrocortisone and 0.125 – 100ng/mL for betamethasone and betamethasone acetate were prepared in an ‘in-house’ artificial synovial fluid. Quality control spiked artificial synovial fluid samples were prepared at 25ng/mL for hydrocortisone and at 20ng/mL for betamethasone and betamethasone acetate. The internal standards were d4-hydrocortisone (at 50ng/mL for hydrocortisone) and triamcinolone acetonide (at 10ng/mL for betamethasone and betamethasone acetate).

**LCMS Conditions**

Sample extracts were analysed in positive ion ESI mode on a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Finnigan, USA) fitted with a high efficiency electrospray ionisation source (HESI). Source conditions were: spray voltage 3010V, vaporizer temperature 350°C, sheath gas pressure 60 units, auxiliary gas pressure 35 units, capillary temperature 300°C, capillary offset 35V. MS/MS conditions for multiple reaction monitoring (MRM) were Q1 peak width 0.2 Da, Q3 peak width 0.7 Da, 0.5 sec/transition, collision gas: argon at a pressure of 1.2mTorr. Precursor ions and their corresponding product ions were as follows: hydrocortisone (collision energy 17eV) m/z 363.2 → 327.2, d4-hydrocortisone (collision energy 17eV) m/z 367.2 → 331.2, betamethasone (collision energy 10ev) m/z 393.2 → 373.2, betamethasone acetate and triamcinolone acetonide (collision energy 10ev) m/z 435.2 → 415.2.

Liquid chromatography was carried out using a Thermo Separations Surveyor™ autosampler and LC pump. The LC column was a Zorbax Stablebond C18, 5µm, 150 x 2mm (Agilent, Australia). Samples (40µL) were injected using a partial loop injection and a 50µL injection loop. Samples were eluted using a linear gradient of 1% acetic acid (A) and methanol (B) at a flow rate of 0.3 mL/min starting at 40%A and 60%B and changing to 5%A and 95%B over 10 min; holding for a further 5 min, returning to initial conditions in 1 min with a final stabilisation time of 6 min. Under these conditions
the analytes had the following retention times: hydrocortisone 5.14 min, d4-hydrocortisone 5.12 min, betamethasone 6.70 min, triamcinolone acetonide 7.13 min and betamethasone acetate 8.43 min.

Results and discussion
The methodology developed for this study showed that betamethasone and betamethasone acetate could be detected in urine and plasma with a limit of detection (LOD) of 0.05 ng/mL and a limit of quantitation (LOQ) of 0.1 ng/mL. Urinary concentrations of betamethasone in the two horses (Beryl and Cheryl) are shown in Figure 1 and the corresponding plasma concentrations are shown in Figure 2. Betamethasone could be detected in urine and plasma for three days and one day, respectively. No betamethasone acetate was detected in either urine or plasma.

No attempt was made to quantify betamethasone sodium phosphate in either urine, plasma or synovial fluid as a pilot experiment had clearly demonstrated that the LOD for this ester was approximately 1 ng/mL and it was not detectable at this level in the earliest samples (1 hour) collected. This is not surprising as the sodium phosphate ester is regarded as a very rapid-acting form of betamethasone and it has been shown in humans to have an exceedingly short half life of 4.7 min (Petersen et al. 1983).

Measurement of betamethasone, betamethasone acetate and hydrocortisone in synovial fluid was complicated by the relatively small volumes of synovial fluid which could be collected. The collection of synovial fluid is an invasive process and in order to minimise any pain and discomfort experienced by the two horses, small volumes only (<100 µL) were withdrawn from alternate legs on alternate days.
Figure 1. Urinary concentrations of betamethasone and hydrocortisone following intra-articular injection of 1mL of Celestone Chronodose® into both radial carpal joints both front knee joints of two horses (Beryl and Cheryl)

Figure 2. Plasma concentrations of betamethasone and hydrocortisone following intra-articular injection of 1mL of Celestone Chronodose® into both radial carpal joints of two horses (Beryl and Cheryl)
Synovial fluid samples were extracted using C18 Omix pipette tips by adapting the methodology developed in this laboratory for the extraction of small (≤100µL) volumes of plasma in pre-race testing applications (Batty et al. in press). This method gave clean extracts and acceptable recovery and reproducibility for the three analytes. The LODs and LOQs were 0.2ng/mL and 0.5ng/mL for betamethasone and betamethasone acetate. Synovial fluid betamethasone and hydrocortisone concentrations are shown in Figure 3. Betamethasone could be detected in synovial fluid for up to 4 days in one horse (Cheryl) and for 3 days in the other (Beryl). Betamethasone acetate was only detected for one day after administration and at greatly differing concentrations in the two horses, 21ng/mL in Cheryl and 2.7ng/mL in Beryl. There was no analytical evidence that the relatively water-insoluble betamethasone acetate persisted in synovial fluid to any measurable degree.

![Figure 3](image)

**Figure 3.** Synovial fluid concentrations of betamethasone and hydrocortisone following intra-articular injection of 1mL of Celestone Chronodose® into both front knee joints of two horses (Beryl and Cheryl)

It has been reported by others that the administration of betamethasone preparations causes a marked fall in endogeneous hydrocortisone concentrations (Popot et al. 2003; Petersen et al. 1983). Suppression of hydrocortisone by exogenous synthetic corticosteroids in horses is well known (Bayer et al. 2001) and has been shown to be particularly profound in the case of triamcinolone acetonide (Teale et al. 2004; Popot et al. 2003).

After IA administration of Celestone Chronodose® suppression of endogenous hydrocortisone is clearly evident for 3-4 days in urine (Figure 1) and 2 days in plasma (Figure 2). The period for which hydrocortisone is suppressed corresponds quite closely to the period over which betamethasone is detectable. This is in agreement with the other studies on betamethasone (Petersen et al. 1983; Popot et al. 2003) but is much less marked than the suppression which occurs after administration of triamcinolone acetonide.

Hydrocortisone was also suppressed in synovial fluid and closely paralleled the suppression occurring in urine and plasma. This would be expected, as synovial fluid is a dialysate of plasma and the concentration of small molecules such as hydrocortisone should be similar to those in plasma.
Comparison of the plasma (Figure 2) and synovial fluid (Figure 3) graphs of hydrocortisone concentration may be interpreted to suggest that its return to normal concentrations may be slightly delayed in synovial fluid compared with plasma implying the possibility of some slight prolongation of effect. Alternatively, this may just reflect the time taken for the equilibration of plasma and synovial fluid hydrocortisone concentrations.

After IA injection of Celestone Chronodose®, betamethasone is detected only at very low concentration (<5ng/mL) for up to 1 day in plasma whereas it is detected for up to 3 days in synovial fluid, albeit at very low concentration (<5ng/mL) on day 3. The higher concentration in synovial fluid may indicate the potential for greater duration of action after IA administration compared with other routes. However, the concentration differences are not very large after the first 24 hours and may not be significant. The data on hydrocortisone suppression may support this latter view, however, this suppression is more likely to depend on systemic betamethasone concentration rather than that in the synovial fluid and by extension in the carpal joint. It is also possible that synovial fluid betamethasone concentrations do not accurately reflect concentrations in the tissues surrounding and within the carpal joint. These tissues could contain higher concentrations of betamethasone acetate which is subsequently hydrolysed and released at very low concentration into synovial fluid thereby contributing to a prolongation of effect.

**Conclusion**

The results of this study may be interpreted to suggest that, on the basis of the analytical results, there may be some support for the claim that the presence of the relatively insoluble betamethasone acetate prolongs the action of Celestone Chronodose® compared with other betamethasone preparations when administered by IA injection. However, any such prolongation of effect would appear to be relatively minor and it is debatable whether this would extend to a period beyond the current urinary detection time of 2-3 days.

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**References**


A study of the intravenous administration of frusemide in the horse

Introduction
Frusemide is a potent loop diuretic commonly administered to racing horses. It acts by inhibiting the reabsorption of electrolytes in the kidney resulting in increased urine production. This leaves less water to be reabsorbed into the bloodstream, decreasing the blood volume. Loop diuretics may also cause a decrease in blood pressure, due to vasodilation of the veins and blood vessels in the kidney. Frusemide is generally administered as a bolus intravenous (IV) injection. Veterinarians use frusemide to treat pulmonary oedema or to increase urine flow rate in horses with acute renal failure. Additionally it is used to in an attempt to prevent exercise-induced pulmonary haemorrhage (EIPH) or “bleeding” in racing horses (Hinchcliff and Muir, 1991 and Stevenson et al., 1994). The diuretic effect of frusemide can reduce the urinary concentration of a range of drugs including acepromazine, clenbuterol, theophylline, fentanyl and morphine. This potential masking effect is of concern to drug control authorities. Diuretics are prohibited substances under AR178 of The Australian Rules of Racing (2006). The purpose of this study was to characterise the pharmacokinetics and post administration detection times following IV administration of frusemide.

Methodology

Administration and sample collection
The administration was carried out by the staff of The University of Queensland, School of Animal Studies, at the Gatton Campus on 10 Standardbred geldings. Each horse underwent a veterinary examination prior to the administration to ensure good health and condition. The horses were exercised every day for 7 days prior to the administration and every day during the administration. Baseline samples of urine and plasma were taken 48 hours, 24 hours and immediately prior to the administration. Each horse was weighed prior to administration. Frusemide (1 mg/kg body weight, Furosemide Injection, Ilium Veterinary Products, Smithfield, Australia) was administered by a bolus intravenous injection using an 18 gauge “Angiocath” intravenous catheter into the right jugular vein. The horses had free access to food and water.

Post administration blood samples were collected from the left jugular vein via a 16 gauge “Milacath” catheter, into 10 mL evacuated lithium-heparinised tubes (Beckton-Dickinson, Rutherford, NJ, USA). Blood samples were collected at 5, 10, 15, 30 and 45 minutes, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 24, 48, 72 and 96 hours post administration. The blood samples were centrifuged at 3000 rpm for 10 minutes and the plasma transferred to plastic screw capped tubes. The plasma samples were stored at –20ºC until analysis.

Urine samples were collected using a customised “Stablemaid” urine collection harness. Urine samples were collected at 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 48, 72 and 96 hours post administration. Total urine voided was recorded for each horse although individual horses did not void urine at every interval. Specific gravity (SG) and pH were recorded for each sample. The urine samples were stored at –20ºC until analysis.

Measurement of Specific Gravity and pH
Urinary SG measurements were made with a portable refractometer (FG-302) calibrated to 1.000 with reverse osmosis/deionised water. The refractometer reading for water was checked between each measurement to ensure stability in the readings. The pH of the urine samples was measured for each sub- aliquot of the urine sample to provide an average pH for the sample.

Analytical Method
Frusemide was quantitatively determined in plasma and urine samples by liquid/liquid extraction followed by liquid chromatography/mass spectrometry (LC/MS/MS) analysis. The method was validated and accepted by NATA as an accredited method under ISO 17025:2005.
All reagents used were analytical grade or better. Water used for the preparation of mobile phase was treated by reverse osmosis (RO) and further by activated carbon and ion exchange filters. Acetonitrile used for the preparation of the mobile phase was HPLC grade (LabScan, Lomb Scientific, Taren Point, Australia). The analytical standard of frusemide (Sigma-Aldrich, Castle Hill, Australia) was used as supplied. Frusemide-d₅ supplied as a certified solution at 200 µg/mL (Neogen Corp, Detroit, MI) was used as supplied.

A 1 mg/mL stock solution of frusemide was prepared by dissolving 25.0 mg of frusemide in ethanol and diluting to 25 mL. This was further diluted with ethanol to produce a working solution at 10 µg/mL. The working internal standard solution was prepared by diluting the supplied solution of frusemide-d₅ to 10 µg/mL. All solutions were stored at 4°C while not in use. Plasma and urine calibration standards were prepared by adding frusemide working solution to drug-free urine and plasma. Plasma calibration standards ranged from 10 to 10,000 ng/mL and urine calibration standards ranged from 10 to 40,000 ng/mL. The calibration standards were prepared daily and run with each batch of samples.

Samples, standards and controls (1 mL) were placed into 16 x 100 mm screw capped tubes. Frusemide-d₅ internal standard working solution (10 µL) was added to all samples, standards and controls. The pH of all samples was adjusted to 5.5 ± 0.2. Samples requiring hydrolysis were hydrolysed overnight at 37°C using β-glucuronidase/arylsulphatase (equivalent to 1000 units Helix pomatia, Boehringer Mannheim). All samples were then extracted with 4 mL ethyl acetate. The ethyl acetate fraction was removed and dried through sodium sulphate. The dry extract was evaporated under nitrogen at 60°C and reconstituted with 100 µL water/acetonitrile (50:50 v/v). The reconstituted extracts were transferred to limited volume sample vials.

Chromatography was carried out by a Waters Alliance HT 2795 using a WATERS Symmetry C18 2.1 mm x 150 mm 5 µm with a PHENOMENEX C18 2.1 mm x 4 mm 5 µm guard column. The mobile phase used was water/acetonitrile/formic acid (50:50:0.01% v/v) isocratic at 300 µL/min. Mass spectrometric analysis was carried out by a Micromass Quattro Micro triple quadrupole mass spectrometer using electrospray ionisation (ESI) in negative mode. Frusemide and frusemide-d₅ were determined by multiple reaction monitoring (MRM) of the transitions 329>285 and 334>290 respectively.
The response for each calibration standard was calculated as a ratio of the frusemide peak area to the frusemide-d₃ peak area. Standard response was plotted against standard concentration to give a calibration curve. Typical calibration curves for plasma and urine analysis are shown in Figure 4.

a)

<table>
<thead>
<tr>
<th>Compound name: Furosemide</th>
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</thead>
<tbody>
<tr>
<td>Coefficient of Determination: R² = 0.999357</td>
</tr>
<tr>
<td>Calibration curve: -2.30646e-006 * x² + 0.242614 * x + -3.11144</td>
</tr>
<tr>
<td>Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)</td>
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<tr>
<td>Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None</td>
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</table>

![Graph for plasma matrix](image)

b)

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
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<tr>
<td>Calibration curve: -3.68876e-007 * x² + 0.109674 * x + 1.5758</td>
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<tr>
<td>Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None</td>
</tr>
</tbody>
</table>

![Graph for urine matrix](image)

Figure 4. Typical frusemide calibration curves for a) plasma matrix and b) urine matrix
**Method Validation**
The method was validated as per the Racing Science Centre Method Validation Procedure Ver. 2.2. The following parameters were determined: instrument linearity, repeatability and detection limit (IDL), method linearity, repeatability, detection limit (MDL), quantitation limit (LOQ), accuracy, recovery and robustness. The results are shown in Table 1.

**Table 1 Method validation parameters for the frusemide quantitation method**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument Linearity (R² Linear Fit / Quad Fit)</td>
<td>0.9991 / 0.9995</td>
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<tr>
<td>Repeatability</td>
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</tr>
<tr>
<td>Detection Limit (IDL)</td>
<td>0.3 ng/mL</td>
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<tr>
<td>Method Linearity (R² Linear Fit / Quad Fit)</td>
<td>0.9999 / 1.0000</td>
</tr>
<tr>
<td>Repeatability</td>
<td>2.3 %</td>
</tr>
<tr>
<td>Detection Limit (MDL)</td>
<td>1.7 ng/mL</td>
</tr>
<tr>
<td>Quantitation Limit (LOQ)</td>
<td>5.3 ng/mL</td>
</tr>
<tr>
<td>Accuracy (Average over 5 concentrations)</td>
<td>13.1 %</td>
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<tr>
<td>Recovery (Average over 5 concentrations)</td>
<td>109 %</td>
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<tr>
<td>Robustness (Placket-Burman)</td>
<td>Pass</td>
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<tr>
<td>Measurement Uncertainty</td>
<td>9 %</td>
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**Pharmacokinetic Analysis**
Intravenous (IV) plasma concentration data was analysed by a non-compartmental method using WinNonLin (Ver. 5.1). The data for Horse 9 was omitted from the calculations because the route of administration could not be modelled using the bolus IV model. Area under the concentration curve (AUC) was calculated for each horse using the linear-log trapezoidal method. The terminal area was estimated and added to the sum of the trapezoids to determine the AUC and the AUC from time 0 to infinity. Maximum concentration (Cₘₐₓ) and Time of Maximum Concentration (Tₘₐₓ) were read directly from the data. Parameters determined from the non-compartmental analysis include terminal phase constant (Kₑ), terminal half-life (T₁/2), apparent volume of distribution (V) and clearance (CL). Results are provided in Table 2.
Table 2. Results and statistics from the pharmacokinetic analysis of the IV plasma data

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>$K_{el}$ (min$^{-1}$)</th>
<th>$T_{1/2}$ (min)</th>
<th>$T_{max}$ (min)</th>
<th>$C_{max}$ (µg/L)</th>
<th>$\text{AUC}_{0-\text{data}}$</th>
<th>$\text{AUC}_{0-\text{inf}}$</th>
<th>CL (L/min)</th>
<th>V (L)</th>
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<td>97.4</td>
<td>5.00</td>
<td>5340</td>
<td>93100</td>
<td>93500</td>
<td>4.92</td>
<td>691</td>
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<tr>
<td>2</td>
<td>0.00626</td>
<td>111</td>
<td>6.00</td>
<td>3980</td>
<td>80300</td>
<td>80400</td>
<td>5.22</td>
<td>835</td>
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<tr>
<td>3</td>
<td>0.00567</td>
<td>122</td>
<td>7.50</td>
<td>3340</td>
<td>70300</td>
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<td>6.47</td>
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<td>81600</td>
<td>81800</td>
<td>5.93</td>
<td>1840</td>
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<table>
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<tbody>
<tr>
<td>Mean</td>
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<td>6.14</td>
<td>4020</td>
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<td>74700</td>
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<td>SD</td>
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<td>49.5</td>
<td>1.35</td>
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<td>12700</td>
<td>12800</td>
<td>1.17</td>
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<tr>
<td>Min</td>
<td>0.00300</td>
<td>97.4</td>
<td>4.80</td>
<td>2330</td>
<td>55200</td>
<td>55300</td>
<td>4.76</td>
</tr>
<tr>
<td>Median</td>
<td>0.00506</td>
<td>137</td>
<td>6.00</td>
<td>3980</td>
<td>78200</td>
<td>78400</td>
<td>5.93</td>
</tr>
<tr>
<td>Max</td>
<td>0.00712</td>
<td>231</td>
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<td>93100</td>
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<td>CV %</td>
<td>30.3</td>
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<tr>
<td>Geometric Mean</td>
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<td>6.02</td>
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<td>73300</td>
<td>73700</td>
<td>5.99</td>
</tr>
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</table>
Results and Discussion
Each sample was analysed with and without enzyme hydrolysis and the excretion curves for plasma and urine are shown in Figures 5 and 6, respectively. There was no apparent difference between the results for hydrolysed and non-hydrolysed samples; this was true for both plasma and urine matrices. This indicates that there were no significant concentrations of conjugated metabolites present in the samples. This may have been due to degradation of metabolites, failure to extract the metabolites or absence of metabolites. It has been reported that, in humans, frusemide is metabolised to frusemide-1-O-acyl-glucuronide (Mizuma et al. 1998). This metabolite is unstable in alkaline urine. The majority of urine samples collected had alkaline pH, indicating that if a glucuronide metabolite had been present, it may have degraded in vivo prior to excretion or on/after collection.

Figure 5. Frusemide concentration in plasma samples over time post administration. The graph is confined to the 0-10 hour range to emphasise the critical data

Figure 6. Frusemide concentration in excreted urine samples over time post administration. The graph is confined to the 0-24 hour range to emphasise the critical data
Examination of the analytical data clearly showed an anomaly in the results from Horse 9. The results for Horse 9 for all sample sets, urine and plasma, hydrolysed and non-hydrolysed exhibited a slow increase in concentration followed by a slow decline in concentration. This is consistent with an inadvertent peri-vascular (PV) injection rather than a bolus intravenous injection. An inadvertent administration of frusemide as PV rather than IV may be caused by a range of factors, including the physiology and temperament of the horse, experience of the veterinarian and the particular situation leading to the requirement of the administration. In this case there was no indication of the PV administration until all the samples had been analysed and examined. The results from Horse 9 are included for information purposes but were not included in the statistical and pharmacokinetic calculations.

The average specific gravity data for all horses in the study (Figure 7) exhibited an immediate decrease after administration with a following steady increase to stabilisation over the next 16 hours. This is a longer stabilisation period than the 8 hours reported by Dirikolu et al. (2003). The horses were given free access to water during the administration, although the amount consumed was not recorded. It is not known how this may have affected the results. The specific gravity results for Horse 9 (Figure 8) showed the same immediate drop as the results for IV administration. However, the SG remains low until hour 5 before it increases to pre-administration levels at hour 16. It is proposed that a PV administration may result in a prolonged effect from the frusemide administration.

Figure 7. Average specific gravity of equine urine samples before and after frusemide IV administration to 9 horses
Figure 8. Specific gravity of urine samples before and after frusemide administration to horse 9

This hypothesis is supported by the urine voided data. Figure 9 shows that Horse 9 voided a significantly larger volume of urine in the first 12 hours and a marginally larger volume over the 12-24 hour period. Figure 10 shows that the IV administrations had a peak void volume in hour 1 then quickly stabilised to a constant volume of approximately 1000 mL per interval. Horse 9 showed a peak volume over the first 5 hours showing an increased effect from the frusemide PV administration.

Figure 9. Graph of total volume of voided urine for each horse over 0-12 hour and 0-24 hour periods
In all plasma sample sets, frusemide was detected up to 48 hours. There was a large range in the C_{max} for the plasma samples from horses receiving frusemide IV. However, the profiles and detection times are very similar. The profile of the PV administration shows the gradual migration of the frusemide into the bloodstream. This profile also supports the data on SG and void volume in indicating a prolonged effect of frusemide within the body. The differences in T_{max} were due to the timing of the first sample rather than actual difference. The pharmacokinetic parameters of AUC0-data, AUC0-inf, clearance and apparent volume of distribution were similar to those determined by Dyke et al. (1996).

In the case of the IV administrations, frusemide was detected in urine samples up to 48 hours, while following PV administration, frusemide was detected for an additional 24 hours. The results for urine are similar to those for plasma (Figure 2) in that the IV samples follow a similar profile and the PV samples show a prolonged excretion.

**Conclusion**

This study has shown that frusemide may be reliably detected up to 48 hours in both blood and urine following IV administration. Urinary void volumes and specific gravity have shown to return to normal values over this period indicating that the diuretic effect would not extend beyond 48 hours.

**Acknowledgements**

The Rural Industries Research and Development Corporation (RIRDC) is acknowledged for funding the horse administration component of this project and the Government of Queensland for funding the analytical component of the project. The enthusiastic participation of the Racing Science Centre Management and Staff in the provision of resources and support is acknowledged and appreciated. The staff of the University of Queensland are also to be commended for their enthusiastic support and professional assistance.
**References**


The detection of triamcinolone acetonide following intra-articular administration in ten horses

Introduction
Ideally, testing for prohibited therapeutic substances in urine taken from competing horses should only detect analytes at concentrations corresponding to physiological distributions that could exert an effect on the animal. Some legitimate therapeutic substances continue to be excreted long after any discernable physiological effect has ceased, and there are grounds for taking measures to exclude such “tail end” detections from punitive action. Racing analysts in such situations need guidance in order to adjust the sensitivity of their testing to ensure it is appropriate for the targeted drug, but determining what urinary concentrations should be applicable has always been a difficult task. Toutain et al. (2002) have presented a model based on the intravenous administration of a drug to come up with an “irrelevant urinary concentration” corresponding to the lowest effectual blood concentration. This is the model adopted by European racing authorities to set reportable concentrations for common veterinary drugs. The Toutain model is relevant only for drugs acting systemically. No such model exists for drugs administered intra-articularly.

The administration of synthetic corticosteroids by any route causes a suppression of the natural endogenous corticosteroid, hydrocortisone (HC), due to biological down-regulation. One possible approach then is to record the period over which this suppression occurs, and to take the concentration of the synthetic corticosteroid at the end of the suppressed period as the analytical cut off value. This method will not work for all corticosteroids as the suppression of HC may not always be significant. However, for triamcinolone acetonide (TA), a commonly used drug in the Australian equine industry for the intra-articular treatment of synovitis/osteoarthritis, it has been demonstrated that the effect is profound (Popot et al. (2003); Teale et al. (2004)). It should be noted that although the suppression of HC is a useful method for determining the duration of effect of TA, it should not itself be used as a criterion for reporting single samples taken as part of doping control measures at race meetings. One reason for this is that there is a delay before the suppression occurs, and samples with both normal concentrations of HC and detectable TA may be due to a TA administration immediately prior to sample collection.

The limited information currently available on the excretion of TA in the horse is based on only small numbers of animals and thus may not truly represent the biological situation. Data from administrations to a larger number of horses will undoubtedly provide a better appreciation of the general equine population. As a result we conducted an examination of the suppression of HC and excretion of TA in ten horses. This information will be invaluable to racing chemists and also to veterinarians, owners, trainers and riders who are frequently required to judge how long after drug administration to wait before returning an animal to competition.

Materials and methods

Horses
Ten thoroughbred geldings (see Table 3 for details) received 2.0 mL Kenacort-A® (Bristol-Myers Squibb; 10 mg/mL TA as an aqueous suspension) via intra-articular injection into the left fetlock joint. Horse T6 did not receive a full dose due to difficulties during injection. It is assessed that possibly 0.5 to 1.0 mL of the dose was wasted. The administrations were conducted in two sessions 2 months apart. In the first session seven horses were involved and urine was collected at -24, 0, 4, 8, and 24 hours relative to the administration, then twice a day to day ten and once a day to day 20. Blood was collected at the same time; however blood samples from day 3 to 7 inclusive were not collected. In the second session 3 horses were involved, and this time both blood and urine was collected at -24, 0, 5, 9, and 24 hours relative to the administration, twice a day until day 10 and then once a day to day 13.

Urine was collected by free void and blood was collected using lithium heparin Vacutainers® (Becton-Dickerson, North Ryde, NSW, Australia).
Table 3. Details of horses used in the study

<table>
<thead>
<tr>
<th>Identification</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
</tr>
</thead>
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<td>648</td>
</tr>
<tr>
<td>T2</td>
<td>12</td>
<td>492</td>
</tr>
<tr>
<td>T3</td>
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<td>T4</td>
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<td>462</td>
</tr>
<tr>
<td>T10</td>
<td>8</td>
<td>532</td>
</tr>
</tbody>
</table>

Solvents and chemicals
TA, HC, and ammonium acetate were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). $^2$H$_4$-HC was obtained from B. Dent Global (Lower Hutt, New Zealand). $^2$H$_6$-TA was synthesized by an in-house method. Dichloromethane and methanol, both Ultra Resi grade, were obtained from J.T. Baker (Phillipburg, NJ, USA). Isopropanol, nano grade, was obtained from Mallinckrodt (Phillipburg, NJ, USA). Glacial acetic acid and sulfuric acid were obtained from Selby Biolab (Clayton, VIC, Australia). High purity water obtained from a Milli-Q system was used for all experiments (Millipore).

Urine extraction
Steroid stripped urine, obtained by passing equine urine through XAD-2 resin packed columns, was used to prepare calibrators and controls. Calibrators were 1.5-150ng/mL for HC and 0.05-5ng/mL for TA. Quality control solutions were 5 and 50ng/ml for HC and 0.1 and 1ng/ml for TA. Duplicate 10mL aliquots of each urine specimen, quality control, calibrator and negative control were pipetted into 25 mL screw-capped tubes. Internal standards $^2$H$_6$-TA (1 ng/mL) and $^2$H$_4$-HC (10 ng/mL) were added to each tube and the solutions were adjusted to pH 9 (±0.5). Dichloromethane (5 mL) was added to each tube; the solutions were mixed for 10 minutes by gentle inversion on a rotorack, and then were centrifuged for 10 min at 2000 rpm. The upper aqueous layer was aspirated to waste before 2 mL of 0.35 M sulfuric acid was added and again gently mixed for 10 minutes. The upper aqueous layer was again aspirated to waste, and the organic layer was evaporated to dryness under oxygen free nitrogen at 60°C. The residues were then reconstituted with 50 μL isopropanol and transferred to autosampler vials for LC-MS analysis.

Plasma extraction
Milli-Q ultra pure water was used to prepare calibrators and controls. Calibrators were 1.5-150 ng/mL for HC and 0.05-5 ng/mL for TA. Quality control solutions were 5 and 50 ng/mL for HC and 0.1 and 1 ng/mL for TA. Duplicate 2 mL aliquots of each plasma specimen, quality control, calibrator and negative control were pipetted into 9 mL screw-capped tubes. Internal standards $^2$H$_6$-TA (1 ng/mL) and $^2$H$_4$-HC (10 ng/mL) were added to each tube. Dichloromethane (4 mL) was added to each tube, and the solutions were mixed for 10 minutes by gentle inversion on a rotorack and then centrifuged for 10 minutes at 2000 rpm. The upper aqueous layer was aspirated to waste, and the organic layer was evaporated to dryness under oxygen free nitrogen at 60°C. The residues were then reconstituted with 50 μL isopropanol and transferred to autosampler vials for LC-MS analysis.

LC/MS Instrumentation
Reconstituted sample extracts were analysed using a Thermo Electron Corporation (San Jose, CA, USA) Finnigan TSQ Quantum Ultra instrument interfaced with a Surveyor LC pump, autosampler and degasser fitted with a 25 μL sample loop. Samples were introduced at a flow rate of 800 μL/min onto an Alltech (Baulkham Hills, NSW, Australia) Alltima C8 column (100 x 4.6 mm, 3 μL particle) and eluted using a solvent gradient. The eluant components were 10 mM pH 4 aqueous ammonium acetate buffer and methanol. The proportion of methanol was increased from 40% to 80% over 6 min and held for 2 min before returning to initial conditions over 0.1 minute. Injection volumes were 5 μL. The MS
was operated in negative ion atmospheric pressure chemical ionization (APCI) mode using selective reaction monitoring (SRM). Analyser conditions were: discharge current 17 µA, vaporiser temperature 450°C, sheath gas 50 arbitrary units, auxiliary gas 10 arbitrary units, capillary temperature 250°C and collision pressure 1.5 milliTorr. Instrument control and processing were performed using Xcalibur software. Acetate adduct precursor ions were monitored for all corticosteroid SRM transitions. Hydroxytriamcinolone acetonide (OHTA) SRM transitions were obtained by performing a full scan product ion experiment on a high level TA administration sample. The resulting ions matched those observed by Teale et al. (2003). The transitions are briefly described below in Table 4.

Table 4. SRM parameters for LC-MS analysis

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<th>Product ion (for qualitative purposes)</th>
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<td>297, 282</td>
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<td>$^2$H$_4$-HC</td>
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<td>not applicable</td>
<td>353, 429, 309</td>
</tr>
</tbody>
</table>

Results and discussion
In the absence of a commercially available OHTA standard it was assumed that it would give a similar response to the parent drug, TA, on monitoring the corresponding SRM transition.

The LOD for the method was defined as the concentration giving a signal to noise ratio of 3. For TA this was determined to be 20 pg/mL, and for HC 100 pg/mL. The LOD for OHTA was assumed to be the same as for TA.
The limit of quantitation (LOQ) for the method was defined as the lowest concentration that could be determined with an acceptable level of uncertainty, and was also the lowest point on the calibration curve. For TA the LOQ was 50 pg/mL and for HC 1.5 ng/mL. Table 5 summarises the precision and accuracy of the method for the analysis of urine samples. Figures 11 to 14 show typical calibration curves.

Table 5. Method quantitation details

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HC in Urine (QC at 5 ng/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average (7/8 results respectively)</td>
<td>5.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.1745</td>
<td>0.3204</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>3.1%</td>
<td>5.5%</td>
</tr>
<tr>
<td><strong>TA in Urine (QC at 0.05 ng/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average (6/8 results respectively)</td>
<td>0.065</td>
<td>0.047</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.0122</td>
<td>0.0047</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>13.6%</td>
<td>10.0%</td>
</tr>
</tbody>
</table>
Results for Urine

Figures 15 - 16 - 17. Concentration versus time graphs of TA, OHTA and HC urinary concentrations in ten horses.
TA was detected in the urine to around 150 hours. OHTA was detected to around 170 hours. The HC remained suppressed from 15 hours post-administration to about 130 hours with the corresponding concentration of TA at that time was an average of 0.16 ng/mL (n=10) (Figures 15-17).

**Results for Plasma**

![TA Excretion](image1)

![Blood HC](image2)

*Figures 18 and 19. Averaged results from the duplicate determinations in a plasma matrix*

In the second experiment, where three horses were used TA in plasma (Figure 18) was detected (on average) to about 72 hours. Hydrocortisone in plasma (Figure 19) was suppressed in two horses for about 120 hours and remained suppressed in the third horse (horse 8) until 180 hours after administration. OHTA was not detected in any of the plasma samples.

**Conclusion**

The intra-articular administration of TA caused a suppression of HC in urine and blood lasting 130 and 120 hours respectively. The urinary concentration of TA at 130 hours was on average 0.16 ng/mL. Hence in order to detect the use of TA in racing animals, urinary screening methods must be capable of detecting TA at concentrations of at least 0.16 ng/mL. Detection of OHTA was possible for up to 170 hours in urine, but was not detected in plasma.

**Acknowledgements**

The Rural Industries Research and Development Corporation (RIRDC) are acknowledged for funding the horse administration component of this study. Racing NSW for funded the analytical part of the project. The staff at CSU and ARFL who did the administrations, collections and analyses are commended for the enthusiasm they showed throughout the project.
References


Implications

This study has provided reliable excretion data for frusemide and triamcinolone acetonide which can be used by veterinarians in estimating appropriate treatment regimes for horses in the weeks or days leading up to racing. The data is of a quality and reliability which will enable it to be shared with similar data for other drugs generated in overseas studies. By contributing to such shared data Australia will reap the benefits of access to that overseas data.

The pilot study of betamethasone and the detailed study of triamcinolone acetonide have demonstrated the feasibility of the research approach taken and have both generated usable data on the excretion of these two drugs after intra-articular administration. The triamcinolone acetonide data which is based on ten horses provides a good guide to the excretion of this substance and will assist racing administrators in evaluating evidence in the event of a positive race day sample.

This study has demonstrated the feasibility of such studies and can be used as a model for further studies. It has also demonstrated the effectiveness of working with relatively large groups of horses in cooperation with the two tertiary institutions, Charles Sturt University and The University of Queensland.

Recommendations

The success of this study has clearly demonstrated the effectiveness and utility of the approach taken to determining reliable drug excretion data. This now needs to be extended to a much wider range of drugs so that equivalent information on a realistic range of therapeutic substances can be made available.

A further application to RIRDC is currently in progress in which it is proposed that studies of 6-10 drugs be carried out on a yearly basis.